



Population genetic structure and delineation of conservation units in European larch (*Larix decidua* Mill.) across its native range

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ABSTRACT

Conservation of the intraspecific genetic diversity of trees is crucial for long-term forest sustainability, especially in the expected scenario of climate change. *Larix decidua* is an excellent example of a very common species for which better knowledge of the population genetic structure is needed. The aim of this study was to examine the molecular genetic variability of *L. decidua* in the Alps and Western Carpathians with adjacent areas using simple sequence repeat (SSR) markers. Low to moderate levels of genetic differentiation were observed among the study populations across the native distribution area of *L. decidua*. Based on AMOVA and PCoA, we revealed a significant correlation between genetic and geographical distances in the whole dataset. The group of analysed populations of Carpathian larch from Eastern Poland, the area of the Jeseníky Mountains in the Czech Republic and the Tatra Region in Slovakia is relatively homogenous with regard to genetics. The F_{ST} values between these populations did not exceed 0.05, indicating low genetic differentiation. In addition, the population from the Eastern Alps could be assigned to this group. A certain level of relationship to Carpathian populations was also observed in the populations from the Northern Alps. In contrast, more significant genetic differences were proven between the individual regions within the analysed populations of Alpine larch from the Southern Alps, including the Maritime Alps, Central Alps, and north-eastern and eastern borders of the Alps. The F_{ST} values between the individual populations from different Alpine regions indicated, in most cases, a medium genetic divergence (0.025–0.112).

Further research is needed to obtain more detailed information about the genetic variability of local native populations of *L. decidua* and their geographical determination, especially in the area of the Alps.

1. Introduction

The importance of forest genetic diversity for forest sustainability is widely recognized (Lefèvre et al., 2013; Rodríguez-Quilón et al., 2016). Maintaining species diversity and habitats has been the main objective of conservation policies to date, while intraspecific genetic diversity has been commonly overlooked (Laikre et al., 2010). The conservation of particular populations within species has been a central task in the European Forest Genetic Resources Programme (EUFORGEN), as most widely distributed forest trees in Europe are not endangered at the species level but often include threatened valuable populations (Lefèvre et al., 2013; Koskela et al., 2013). Rodríguez-Quilón et al. (2016) highlighted that the identification of conservation units below the species level should account for key neutral and adaptive components of genetic diversity, especially in species with strong population

structure and complex evolutionary histories.

L. decidua is an excellent example of a highly variable common forest species responding to changing ecological conditions. An investigation of various morphological traits confirmed the clinal character of its geographical variability, causing challenges in the description of intraspecific variability (Foffová, 2013). Despite these challenges, this aspect of the population genetics of *L. decidua* has received relatively little attention. Very few studies have investigated the range-wide genetic differentiation in European larch. Most of the available studies were conducted using isoenzymes and give only an approximate, undetailed picture of the general intraspecific variability of European larch (Belletti et al., 1997; Mihai and Teodosiu, 2009), sometimes with contradictory results. For example, Lewandowski and Mejnartowicz (1991) concluded that there is no statistically significant differentiation between stands and that the genetic differences are not

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related to geographic distance. At the same time, the results of Maier (1992) show a general correlation between genetic and geographic distances.

The need for increased resolution to better understand the population genetic structure and the past population dynamics of *L. decidua* has led to the use of DNA markers. Mosca et al. (2014), based on their findings in the Alpine region, assume that the genetic structure of larch populations could be affected by both geographical isolation and environmental gradients, creating opportunities for local adaptation. The findings of Pluess (2011) from the Aletsch valley in the Swiss Alps indicate that intensive mixing of the genes in the *L. decidua* population, which is expanding rapidly as a consequence of climate warming, can maintain levels of genetic diversity with no indication of founder events. Nardin et al. (2015) observed low genetic differentiation between larch stands along the altitudinal gradient in the French Alps and considered them a single population. The genetic structure of populations of European larch is determined by geographical isolation and climate but also by human impact, including human-induced translocations and breeding that have changed the genetic make-up of extant populations (Mosca et al., 2014; Wagner et al., 2015a,b; Schulze et al., 2007; Garbarino et al., 2009; Pâques et al., 2013; Jansen and Geburek, 2016). This situation raises conservation concerns regarding native populations.

The main aim of this study was to provide a more comprehensive picture of the molecular genetic variability of *Larix decidua* across its native distribution area and to compare its genetic parameters with the described distribution of currently distinguished intraspecific units (ecotypes) mentioned by Foffová (2013). Simple sequence repeat (SSR) markers were employed for analysis. The data obtained can provide critical information for the determination of the genetic conservation units of native populations of European larch and serve as a basis for genetic conservation strategy. Using a set of samples collected across its native distribution area, we aim (i) to evaluate the genetic variability of European larch and (ii) to determine the extent to which genetic discontinuities exist in different parts of the native distribution range.

2. Materials and methods

2.1. Study species

Larix decidua Mill. is a medium-sized to large monoecious deciduous and wind-pollinated coniferous tree, reaching 25–45 m tall, with a trunk up to 1 m in diameter. The pollen grains are small and lack sacchi. For that reason, they are transported by the wind for relatively short distances, up to 300 m. Larch trees reach sexual maturity at the age of 15 years in open stands and 35–40 years in closed stands (Matras and Pâques, 2008), but their life span can reach 400 years (Carrer and Urbinati, 2004). Larch trees produce seeds every 3–4 years on average with a seed life span ranging from 3 to 7 years. The seeds are mostly dispersed by wind and birds (Rameau et al., 1993).

The native distribution area of *Larix decidua* is relatively small, disjunctive and limited to the territory of Central Europe, where it currently occurs in the Alps, in the foothills of the Jeseníky Mountains close to the north-eastern border of the Czech Republic (Sudeten) and in the Western Carpathians (North Slovakia and adjacent areas in South-eastern Poland). Some small outstanding native populations have also been recorded in Central Poland and the Romania Carpathians (for details, see Meusel et al., 1965; Pâques et al., 2013). However, the distribution area of *L. decidua* has changed since the last glacial period and the beginning of the Holocene (Wacnik et al., 2004; Jankovská and Pokorný, 2008). The native range-changes of *L. decidua* over the last interglacial-glacial cycle in the context of long-term and short-term climate changes and anthropogenic impact are documented in fossil compilations (Wagner et al., 2015b; Jankovská, 2007). In this context, the seven most recent glacial disjunctive refuges were identified by genetic data (Wagner et al., 2015b).

Currently, the species *L. decidua* is mainly divided into two subspecies (Franco et al., 1964; Jasičová, 1966; Boratynski, 1986; Skalický and Skalický, 1988): (i) the nominate subsp. *decidua*, inclusive of native populations from the Alps, Jeseníky Mountains, Slovakia and Romania, and (ii) the subsp. *polonica* (Racib.) Domin, which is related only to larches from lower altitudes in Poland. Farjon and Filer (2013) differentiate only three taxa under the name *L. decidua* at the variety level: var. *decidua* (distribution in the Alps and the Carpathians), var. *carpathica* Domin (distribution in the Carpathians) and var. *polonica* (Racib. ex Wóycicki) Ostenf. & Syrach (distribution in Poland).

Foresters prefer to divide the variability of *L. decidua* according to geographical region or regional population (ecotypes); for details, see Foffová (2013). This approach supports the division of the provenances of *L. decidua* into two groups: Alpine larch and Carpathian larch (including the native populations from the Jeseníky Mountains, Slovakia, Poland and Romania). Within the Alpine larch group, the following four ecotypes are distinguished, differing mainly by the level of adaptation to the climatic conditions of mountainous sites, corresponding to particular areas of contemporary geographical distribution: 1. Italian larch – *italica* (the Southern Alps including the Maritime Alps), 2. Raetic larch – *raetica* (the Central Alps), 3. Tyrolian larch – *tirolica* (the Northern Alps), and 4. Nordic larch – *norica* (north-eastern and eastern borders of the Alps). The Carpathian larch is split into four smaller particularly isolated regions and separate remnant stands in the Romanian Carpathians. The following four ecotypes were distinguished: 1. Sudeten or Silesian larch – *sudetica* (the Jeseníky Mountains, the Czech Republic), 2. Polish larch – *polonica* (Poland), 3. Slovac or Tatra larch – *slovacica* (the Western Carpathians), and 4. Romanian larch – *romanica* (the Eastern and south Carpathians).

2.2. Plant material and DNA isolation

During the growing seasons of 2009–2012, we collected samples from 21 populations of *Larix decidua* in their natural habitats across the area of the natural distribution of the species (Table 1). At each locality, samples were taken usually from 50 (min. 31; max. 55) trees, which we believe to constitute a sufficiently representative sample of the population. Several healthy brachyblasts with needles were taken from each exemplar. If the tree height interfered (populations in Poland) with collecting needles, a shotgun was used to down the branches. Samples were then put into plastic bags and kept in a cooling box until they were delivered to the laboratory. Then, the fresh and healthy needles were frozen at –25 °C. Prior to DNA extraction, the samples were frozen at –196 °C, lyophilized and ground with glass beads in a Retch shaking mill. Total DNA was extracted using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

2.3. Employed markers and molecular genetic analysis

Specific SSR markers were not yet available when the study was conducted. For this reason, we chose the method of cross-species amplification. Eight nuclear SSR primers originally described for another *Larix* species were used for this study – UAKLy2, UAKLy6, UAKLy7, and UAKLy10a (Khasa et al., 2000) and bCLK253, bCLK263, bCLK228, and bCLK211 (Isoda and Watanabe, 2006). Amplification reactions were performed according to the procedures described by Khasa et al. (2000) and Isoda and Watanabe (2006) with minor modifications. PCR was performed on a thermal cycler GeneAmp PCR System 9700 (PE Applied Biosystems). DNA fragments were separated on 6% denaturing polyacrylamide gels (sequi-Gen GT System, Bio-Rad) and visualized by silver staining according to the Promega Silver Sequence DNA Sequencing System as modified by van der Schoot et al. (2000). The sizes of the PCR products were determined by comparison to an accompanying sequencing reaction using pGEM®-3Zf(+) control DNA (Promega).

Table 1
Overview of the studied *Larix decidua* populations.

Population no.	Latitude	Longitude	Altitude [m]	Locality name	Country	Number of analyzed individuals	Sampling date
1	44° 12' N	07° 14' E	1,717	Terme di Valdieri; Refugio Regina Elena A.N.A.	Italy	48	11.7.2011
2	44° 31' N	07° 08' E	1,730	Morinesio	Italy	50	15.8.2009
3	44° 30' N	07° 08' E	1,580	Val Po; Chianale	Italy	48	12.7.2011
4	44° 37' N	07° 01' E	1,750	Val Po; Maddalena	Italy	48	15.8.2009
5	46° 01' N	07° 07' E	1,050	Val d'Arpette	Switzerland	50	13.6.2010
6	45° 52' N	07° 49' E	1,925	Gressoney la Trinité	Italy	55	13.8.2012
7	46° 23' N	08° 01' E	1,800	Bitch; Stausee Gibidum	Switzerland	50	11.6.2010
8	46° 36' N	09° 31' E	1,347	Schmitten	Switzerland	42	27.6.2011
9	47° 34' N	13° 35' E	1,479	Gossau-Plassen; Rossalm	Austria	50	17.6.2010
10	47° 32' N	13° 31' E	1,589	Gossau; Modereck	Austria	48	16.6.2010
11	47° 36' N	13° 59' E	1,623	Tauplitz; Tauplitzalm	Austria	48	5.6.2011
12	47° 35' N	15° 14' E	1,700	Hochschwab	Austria	31	30.6.2012
13	50° 18' N	17° 06' E	482	Borový Nature Reserve	Czech Republic	50	27.8.2009
14	50° 09' N	17° 20' E	915	Suchý vrch Nature Reserve	Czech Republic	50	27.8.2009
15	50° 02' N	17° 33' E	450	Ptačí hora Nature Reserve	Czech Republic	45	21.7.2011
16	49° 53' N	17° 31' E	774	Velký Roudný Nature Reserve	Czech Republic	50	28.8.2009
17	49° 07' N	20° 03' E	1,350	Štrbské pleso (High Tatra Mountains)	Slovakia	50	2.6.2009
18	48° 43' N	19° 59' E	980	Voniaca (Muráňská planina Mountains)	Slovakia	50	3.6.2009
19	49° 07' N	20° 50' E	520	Brezovička	Slovakia	49	2.6.2009
20	51° 09' N	20° 41' E	338	Suchedniów (Ciestowice Nature Reserve))	Poland	50	30.6.2009
21	51° 03' N	20° 45' E	346	Suchedniów (Obreb Bliżyn)	Poland	50	30.6.2009

2.4. Data processing

The standard parameters of genetic variability were determined: number of alleles, observed heterozygosity (H_o) and expected heterozygosity (H_e). For each population, the Wright's fixation index F_{IS} was examined to evaluate the deviation from Hardy-Weinberg equilibrium, which allowed us to estimate the relevance of inbreeding (AMOVA procedure, 1000 permutations). The calculation of genetic differences between populations was based on 31–55 individuals from each population (Table 1). The genetic distance between populations was estimated by the statistic F_{ST} (Wright, 1951) using the program Arlequin 3.1 (procedure AMOVA) (Excoffier et al., 2006) and visualized by PCoA using GenAlEx 6.502 (Peakal and Smouse, 2006). Pairwise geographical distances were measured using Tools for ArcGIS. The relationship between genetic and geographical distances was tested by linear regression, and the significance of the correlation coefficient was verified by the Mantel test. Differences in the regression coefficients of the populations from the Carpathians and Southern Alps were tested by the algorithm described in Diem (1960). This algorithm tests the hypothesis of identity of two regression coefficients using Student's t-distribution. The software program STATISTICA v. 9.0 (StatSoft Inc., 2009) was used for the statistical analyses.

Groups of genetically related populations were differentiated using the software STRUCTURE version 2.3 (Pritchard et al., 2010). The number of clusters (K) was set from 1 to 22 (i.e., number of populations + 1), with 10 runs for each K value. We checked the necessary number of burn-in iterations and found a high degree of convergence with 100,000 iterations, as recommended by Gilbert et al. (2012). We used 100,000 iterations of burn-in and after burn-in and explored the admixture model. To determine the most likely value of K, we used Evanno's method in the software STRUCTURE HARVESTER (Evanno et al., 2005; Earl and von Holdt, 2012; Wagner et al., 2015a).

3. Results

The genetic diversity across all populations was moderate, with an H_e of 0.686 (range = 0.602–0.768). The values of Wright's fixation coefficient (F_{IS}) ranged between -0.018 and 0.245. F_{IS} was not significant in two populations from Austria (-0.018 and -0.006; localities Nos. 9, 10) and in one population from Switzerland (0.037; locality no. 8) – for localities, see Table 1. All of the other eighteen populations differed from Hardy-Weinberg equilibrium, indicating a small

proportion of inbreeding (F_{IS} ranged between 0.105 and 0.245). The standard genetic diversity indices per population are summarized in Supplementary Material 1.

3.1. Genetic differentiation among populations

Low to moderate levels of genetic differentiation were observed among the study populations across the native distribution area of *L. decidua* (Supplementary Material 2). The F_{ST} values ranged from 0.015 between population Nos. 13 and 14 (both with the Jeseníky Mountains) to the statistically significant ($p < 0.05$) value of 0.150 between population Nos. 6 (the Central Alps) and 15 (the Jeseníky Mountains) – for localities, see Table 1. Principal coordinate analysis shows the genetic differentiation of the study populations in different locations (Fig. 3). The Mantel test (Fig. 1) revealed a significant correlation between genetic and geographical distances in the whole dataset ($R^2 = 0.664$; $p = 0.0001$). The relationship between geographic and genetic distance remained significant when populations from the Carpathians (locality Nos. 13–21) and from the comparably large region in the Southern Alps and western part of the Central Alps (locality Nos. 1–7) were analysed separately. However, the Alpine populations showed a significantly steeper increase in genetic distance with geographic distance than the Carpathian populations ($b = 19.1 \times 10^{-5}$ vs. $b = 3.2 \times 10^{-5}$; $p = 0.02$). The results mentioned above and the F_{ST} values visualized in Fig. 3 indicate that the group of analysed populations of Carpathian larch from Eastern Poland, the area of the Jeseníky Mountains in the Czech Republic and the Tatra Region in Slovakia is relatively homogenous with regard to genetics. The F_{ST} values did not exceed 0.05 (0.015–0.050), showing low genetic differentiation. In addition, the population from the Eastern Alps could also be assigned to this group (locality No. 12; for locality see Table 1).

In contrast, Fig. 3 shows more significant genetic differences between the individual regions within the analysed populations of Alpine larch (the Southern Alps including the Maritime Alps, the Central Alps, the Northern Alps, and the north-eastern and eastern borders of the Alps). The F_{ST} values between the individual populations from different Alpine regions showed medium genetic divergence (0.025–0.112) in most cases. In isolated cases, values indicating medium genetic divergence were also recorded within the region of the Southern and Central Alps. The populations of the southern and eastern areas of the Central Alps (locality Nos. 6 and 8) are genetically less differentiated from the population of the Southern Alps than from the populations of the north-

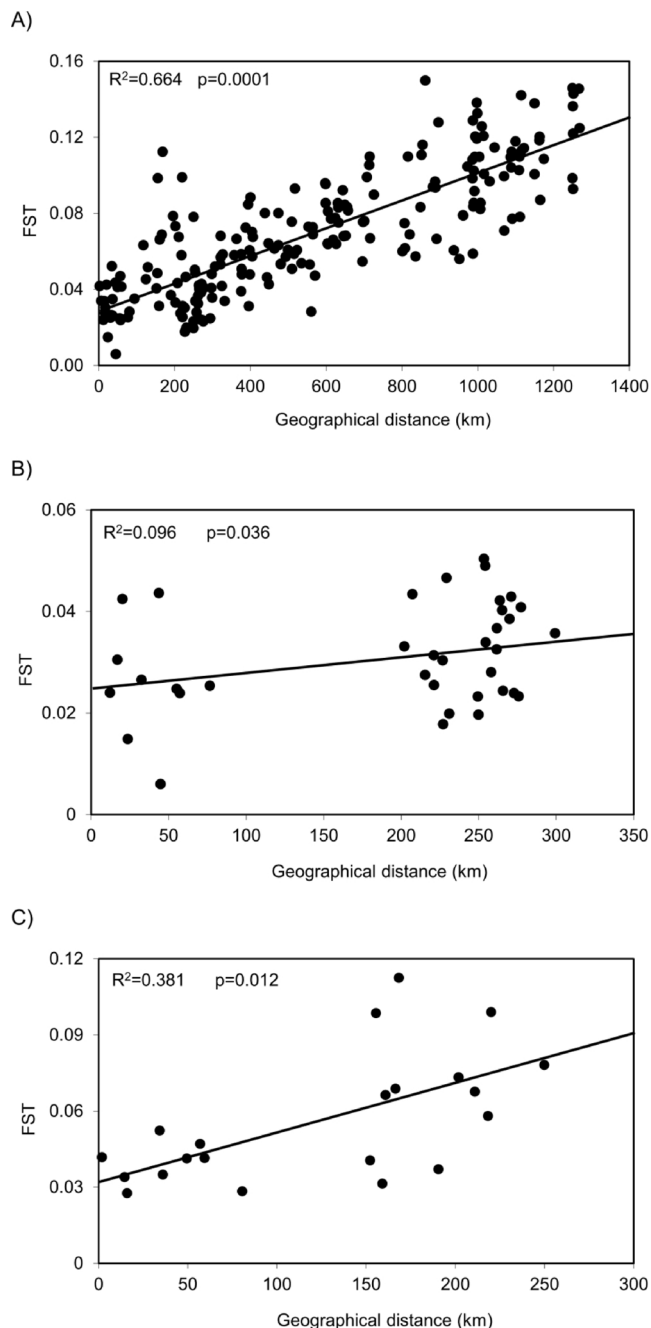


Fig. 1. Association between pairwise geographical distances and pairwise F_{ST} values (Mantel test). A – all 21 studied populations of *Larix decidua*; B – the Carpathians; C – the Southern Alps and the western part of the Central Alps.

western part of the Central Alps (locality Nos. 5 and 7; for localities see Table 1).

The Bayesian analysis of the population structure supported differentiation into two or five clusters (Supplementary Material 3). Studies using Evanno's method frequently identify two groups ($K = 2$) as the top level of hierarchical structure, even when more subpopulations are present (Janes et al., 2017). For this reason, we selected the solution of five groups ($K = 5$) with a high assignment of individuals to one of three defined groups for Alpine larch and to two groups for Carpathian larch (Supplementary Material 4). Figs. 2 and 3 document readily distinguishable differences between the populations from the Southern, Central, and Northern Alps. However, the results do not show genetic differentiation among the populations from Poland, the Jeseníky Mountains (the Czech Republic) and the Tatra Region (Slovakia). Fig. 2

also shows the limited differentiation of the population from the south and eastern part of the Central Alps with regard to the populations of the Southern Alps and populations from the north-western part of the Central Alps.

The results also show that population No. 12 from the eastern borders of the Alps (see Table 1) has a boundary position between the populations of the Northern Alps and the group of genetically non-differentiated ecotypes of Carpathian larch.

4. Discussion

The genetic diversity of European larch observed in our study ($H_e = 0.686$) was as high as expected in boreal and temperate conifer species (e.g., Hamrick et al., 1992; Nybom, 2004). This result is consistent with the data for *L. decidua* detected using SSR analysis by Pluess (2011) from the Swiss Alps ($H_e = 0.75$) and Nardin et al. (2015) from the French Alps ($H_e = 0.761$).

4.1. Genetic variation among populations of *Larix decidua*

The analysis of nuclear DNA data of European larch from populations collected in their natural habitats across the area of its natural distribution revealed genetic differentiation associated with geographic distances but did not completely reflect the current taxonomic division and distribution of distinguished ecotypes as described, for example, by Farjon and Filer (2013) and Foffová (2013). Our results confirmed the genetic differentiation between the areas assigned to the occurrence of individual distinguished ecotypes of Alpine larch but did not confirm the genetic differentiation between the ecotypes of Carpathian larch. However, Lewandowski and Mejnartowicz (1991) described generally low differentiation among the populations of European larch. As emerged from our analysis, the sampled populations of Carpathian larch from the Czech Republic, Poland and Slovakia are the most homogenous. It seems that the differentiation of three distinguished intraspecific taxa in this region, among which *Larix decidua* var. *polonica* and *L. sudetica* are included in the Red Lists (Farjon and Filer, 2013; Procházka, 2001), is questionable.

In addition, the population from the Eastern Alps differed only slightly from the group of Carpathian larch and, according our analysis, could be assigned to this group. A certain level of relation to this group was also shown by the populations from the Northern Alps. Maier (1992) reached a similar conclusion using an isozyme analysis.

The relationship between the populations from the Eastern Alps and the populations of Carpathian larch could indicate the postglacial recolonization of both regions from one larger refuge between the Alps and Carpathians, rather than the several small scattered refuges that were assumed by Wagner et al. (2015b) based on their results. The population in the eastern part of the Alps could then have been influenced by recolonization and introgression by the populations from the southern or western refuges in the foothills of the Alps. Wagner et al. (2015a), however, found a low degree of differentiation in the population from the Czech Republic, Poland and Slovakia; however, they still distinguished two groups of populations within this region, one from the Czech Republic and one from Slovakia and Poland. In contrast, they did not find any more significant differences between the populations from the Northern Alps and the eastern border of the Alps and assigned them to the Alpine larch. However, the extent to which the populations, especially in the Eastern Alps and Carpathians, were influenced by within-range translocations remains an unanswered question.

The results of our analysis also showed that the sampled populations of Alpine larch from the Southern and Central Alps could be assigned to two groups. The populations from the north-western part of the Central Alps in Switzerland and the populations from the Southern Alps are relatively well differentiated; however, the populations from the southern and eastern part of the Central Alps are related to the

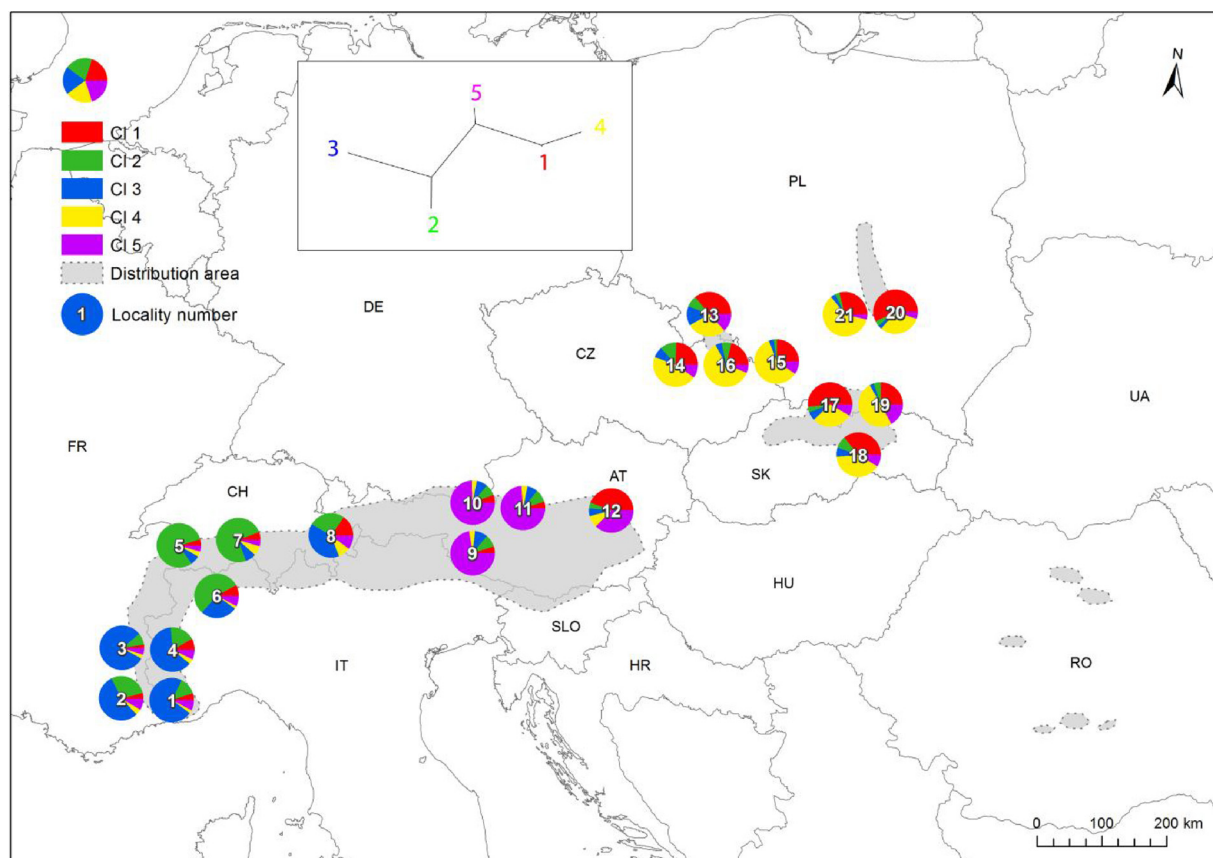


Fig. 2. Neighbour joining tree and distribution of the five SSR nuclear clusters detected by STRUCTURE in studied populations of *Larix decidua*. For detailed information about the localities marked in the diagrams, see Table 1. Distribution data based on Meusel et al. (1965) and Foffová (2013).

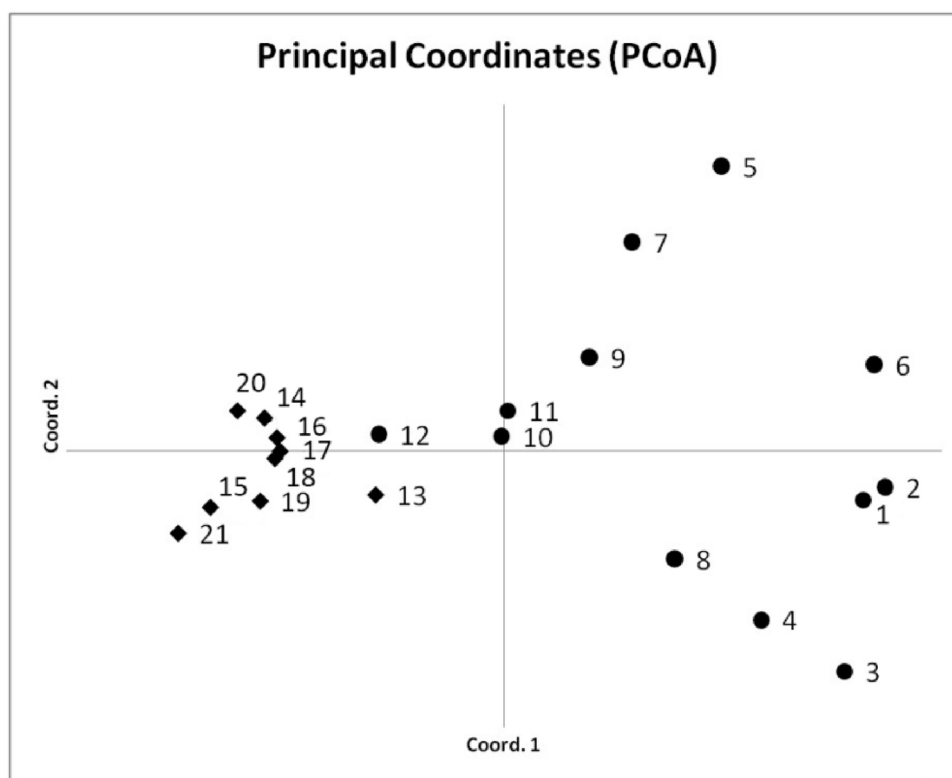


Fig. 3. Principal coordinate analysis (PCoA) visualization based on F_{ST} values for 21 studied populations of *Larix decidua*. Circles – Alpine larch; diamonds – Carpathian larch.

For localities, see Table 1 in the text. For F_{ST} values, see Supplementary Material 2.

populations from the Southern Alps. This relationship may indicate two postglacial recolonization pathways of the Central Alps from the refuges in the northern and southern foothills. In areas where the geomorphology of the Alpine mountain range enabled populations from northern and southern recolonization pathways to meet, a two-way introgression of the two genetically different populations probably occurred. Wagner et al. (2015a, Wagner et al., 2015b) reported the genetic differentiation between the populations from the Southern and Central Alps; however, they did not demonstrate a difference between the populations from the north-western and south-eastern parts of the Central Alps. A comparison of the results showed that the genetic variation among the populations that were genetically distant in the Alpine area, representing the focal point of European larch distribution, was significantly higher than that among the relatively small, isolated populations of the disjunctive subarea in Central Europe (the Czech Republic, Slovakia and Poland). This result may be related to landscape shape (orography) that allows different levels of connectivity among locations, which might be more important than the environmental gradients described by Mosca et al. (2014). The higher genetic differentiation in the Alps could also be a result of genetic differentiation in more Alpine refuges during the glacial period. Wagner et al. (2015b) assumed three refuges located south of the Alps and three located in eastern Central Europe. Our results indicate the possibility of more refuges in the foothills of the Alps and the possibility that the populations from Central Europe originated from only one refuge. The genetic differentiation of populations therefore may not be directly related to geographical distance.

5. Conclusion and conservation implications

Low to moderate levels of genetic differentiation were observed among the study populations across the native distribution area of *L. decidua*. The group of analysed populations of Carpathian larch (from Eastern Poland, the area of the Jeseníky Mountains in the Czech Republic, and the Tatra Region in Slovakia) is relatively homogenous with regard to genetics. The local native populations of this region could be treated as one conservation unit. In contrast, more significant genetic differences were proven between the individual regions within the analysed populations of Alpine larch. Analyses of population structure indicated three groups within the Alpine larch that corresponded to the areas of the Southern Alps, including the Maritime Alps, the Central Alps and the Northern and Eastern Alps. The last group showed a certain level of relation to Carpathian larch. Our findings support the existence of distinguished intraspecific taxa (ecotypes) in the territory of the Alps (see Foffová, 2013). This implies that the local native populations from the areas of the Southern Alps, including the Maritime Alps, the Central Alps and the Northern and Eastern Alps, should be treated at least as three different conservation units. However, the results of Mosca et al. (2014) indicate that the genetic variability of European larch in the Alps is probably more complicated.

Further research is needed to clarify the genetic variability of local native populations of *Larix decidua* and their geographical determination, especially in the area of the Alps. In the future, such data will be essential for the creation of a conservation unit network of European larch, which requires additional conservation focus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.flora.2018.06.007>.

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